

RESEARCH ARTICLE

The Orphan Nuclear Receptor ERR γ Regulates Hepatic CB1 Receptor-Mediated Fibroblast Growth Factor 21 Gene Expression

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Abstract

Background

Fibroblast growth factor 21 (FGF21), a stress inducible hepatokine, is synthesized in the liver and plays important roles in glucose and lipid metabolism. However, the mechanism of hepatic cannabinoid type 1 (CB1) receptor-mediated induction of FGF21 gene expression is largely unknown.

Results

Activation of the hepatic CB1 receptor by arachidonyl-2'-chloroethylamide (ACEA), a CB1 receptor selective agonist, significantly increased *FGF21* gene expression. Overexpression of estrogen-related receptor (ERR) γ increased *FGF21* gene expression and secretion both in hepatocytes and mice, whereas knockdown of ERR γ decreased ACEA-mediated *FGF21* gene expression and secretion. Moreover, ERR γ , but not ERR α and ERR β , induced *FGF21* gene promoter activity. In addition, deletion and mutation analysis of the *FGF21* promoter identified a putative ERR γ -binding motif (AGGTGC, a near-consensus response element). A chromatin immunoprecipitation assay revealed direct binding of ERR γ to the *FGF21* gene promoter. Finally, GSK5182, an ERR γ inverse agonist, significantly inhibited hepatic CB1 receptor-mediated *FGF21* gene expression and secretion.

Conclusion

Based on our data, we conclude that ERR γ plays a key role in hepatic CB1 receptor-mediated induction of *FGF21* gene expression and secretion.

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Introduction

The three estrogen-related receptors (ERRs), termed α , β , and γ , belong to the NR3B subfamily of the nuclear receptor superfamily. ERRs bind to the estrogen response element as dimers or to the half-site core sequence (TNAAGGTCA) as monomers. ERR isoforms are expressed in the pancreas, heart, brain, and liver [1–3]. ERR γ plays important regulatory roles in various metabolic events. ERRs are regulated by the peripheral circadian clock in key metabolic tissues, such as muscle, white or brown adipocytes, and liver [4]. ERR γ plays an essential role in the maturation of glucose-response β -cells [5]. In brown adipose tissue, ERR γ induces Uncoupling Protein 1 (UCP1) expression and fatty acid oxidation [6]. It is also important in cancer therapy, where it is used as a marker of clinical course and in the selection of appropriate therapies [7]. ERR γ suppressed tumor growth and the proliferation of prostate cancer cells [8]. We also reported that ERR γ is involved in insulin-mediated inhibition of hepatic gluconeogenesis [9]. In addition, GSK5182 controls ERR γ -induced hepcidin gene expression and improves *Salmonella typhimurium* infection by modulating host iron homeostasis [10]. Previously, we demonstrated that hepatic ERR γ regulates the expression of gluconeogenic genes and blood glucose levels in a mouse model of type 2 diabetes and plays a key role in hepatic insulin signaling mediated by lipin1 [11, 12]. Moreover, ERR γ displays endogenous ligand-independent constitutive transcriptional activity that depends on its interaction with coactivators or corepressors. PKB/Akt suppresses the transcriptional activity of ERR γ by promoting the phosphorylation of ERR γ at S179 and by eliciting translocation of ERR γ from the nucleus to the cytoplasm [9]. GSK5182, a 4-hydroxytamoxifen analog, is a selective inverse agonist of ERR γ [13]. GSK5182 inhibits ERR γ transcriptional activity increasing the interaction between ERR γ and the corepressor of SMILE [14].

The endocannabinoid system, which consists of two G protein-coupled receptors, CB1 and CB2 (cannabinoid receptor type 1 and 2), is an endogenous lipid signaling pathway. Anandamide and 2-arachidonyl glycerol (2-AG) are the two best characterized endogenous cannabinoid activators of CB1 and CB2 [15]. The CB1 receptor is expressed in the brain, vascular tissues, heart, and liver, whereas the CB2 receptor is expressed in most immune cells. 2-AG synthesis is achieved through the hydrolysis of diacylglycerol (DAG) by DAG lipases (DAGL α and DAGL β) [16, 17]. Activation of the hepatic CB1 receptor promotes fatty acid synthesis and diet-induced obesity [18, 19]. The synthesis of 2-AG is promoted by alcohol-mediated upregulation of DAGL β in hepatic stellate cells. 2-AG activates the CB1 receptor on adjacent hepatocytes by a paracrine mechanism [20]. Arachidonyl-2'-chloroethylamide (ACEA) is a synthetic CB1 receptor selective agonist [21], while AM251 is a CB1 receptor selective antagonist [22]. Previously, we reported that activation of the CB1 receptor inhibits insulin receptor signaling through cAMP-responsive element-binding protein 3-like 3 (CREBH)-mediated lipin1 gene expression in the liver [23]. We also reported that ERR γ regulates cytochrome P450 2E1 (CYP2E1) expression and oxidative liver injury by alcohol via the hepatic CB1 receptor [24].

Fibroblast growth factor (FGF) 21 is a member of the FGF family [25] and is a metabolic hormone secreted predominantly by hepatocytes [26]. Unlike the classical members of the FGF family, FGF21 does not have heparin-binding properties, which enables its release into the circulation [27]. Therefore, FGF21 acts through cell surface receptors composed of classic FGF receptors complexed with β -Klotho [28]. FGF21 regulates carbohydrate and lipid metabolism. For example, FGF21 increases glucose uptake via the induction of glucose transporter 1 in adipose tissue [29]. FGF21 also increases fatty acid oxidation and gluconeogenesis in the liver [30–32]. FGF21 protects against acetaminophen (APAP)-induced hepatotoxicity by increasing the peroxisome proliferator-activated receptor coactivator (PGC)-1 α -mediated antioxidant capacity [33]. FGF21 expression is increased in the liver in the fasting condition by activation

of the nuclear receptor peroxisome proliferator-activated receptor (PPAR) α . It is also increased in the fed condition to regulate PPAR γ in adipose tissue [31, 34]. Recently, the nuclear receptor retinoic acid receptor-related orphan receptor α (ROR α), retinoic acid receptor β (RAR β), and farnesoid X receptor (FXR) were shown to play a role in regulating *FGF21* in the liver [35–37]. Moreover, growth hormone receptor (GHR) signaling directly stimulates *FGF21* gene transcription in the liver by janus kinase 2 (JAK2)-signal transducer and activator of transcription 5 (STAT5) [38]. However, the mechanism of hepatic cannabinoid type 1 (CB1) receptor-mediated induction of *FGF21* gene expression is largely unknown.

Previously, we reported that activation of the CB1 receptor induces *CREBH* gene expression and its transcriptional activity in the liver [23], and that *CREBH* regulates *FGF21* promoter activity [39]. We also found that hepatic *ERR γ* expression is induced by ethanol via the activation of CB1 receptor signaling [24]. In this study, we demonstrated that the orphan nuclear receptor *ERR γ* is responsible for CB1 receptor-mediated *FGF21* expression. Hepatic *ERR γ* gene expression is induced by activation of the CB1 receptor, and knockdown of *ERR γ* gene expression prevents CB1 receptor-mediated *FGF21* expression. Moreover, *FGF21* gene expression and secretion are inhibited by an inverse agonist of *ERR γ* . Collectively, this study demonstrates that *ERR γ* is a novel regulator of *FGF21* gene expression and secretion.

Materials and Methods

Ethics Statement

Animal experiments were approved by the Chonnam National University Animal Care and Use Committee (No. CNU-IACUC-YB-2014-39).

Chemicals

ACEA was purchased from Tocris Bioscience. GSK5182 was synthesized as described previously [13]. GSK5182 was used at a concentration of 10 μ M *in vitro* and 40 mg/kg in mouse experiments.

Animals

Male 8-week-old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used for this study. Mice were maintained at 24°C on a 12:12 h light-dark cycle. CB1 receptor-knockout (CB1^{-/-}) mice were kindly provided by Dr. George Kunos at the National Institute on Alcohol Abuse and Alcoholism/NIH as described previously [40, 41]. Eight-week-old male CB1^{-/-} mice were used to obtain primary hepatocytes. All animals were allowed *ad libitum* access to food and water. For the compound studies, ACEA administration (10 mg/kg, intraperitoneal injection) was performed in wild-type mice. Ad-GFP and Ad-FLAG-*ERR γ* were injected via the tail vein, and mice were sacrificed on day 3 after the injection. To identify the effect of *ERR γ* , control and recombinant sh*ERR γ* adenoviruses were injected into mice in the presence or absence of ACEA (10 mg/kg, intraperitoneal injection). GSK5182 was administered by intraperitoneal injection (40 mg/kg). Liver tissues from chronic alcohol diet mice were used to measure *FGF21* gene expression as described previously [24]. Briefly, alcohol was administered for 4 weeks (chronic alcohol model) and GSK5182 was given by oral gavage administration once-daily for the last 2 weeks of alcohol feeding. The mice were monitored once-daily after experimental injection. Mice were injected with Rompon (BAYEL) and Zoltil50 (Virbac) and sacrificed by exsanguination according to the protocol of the Chonnam National University Animal Care and Use Committee (No. CNU-IACUC-YB-2014-39).

Plasmids and DNA constructs

The FGF21-Luc (-2078bp/+129bp) construct was described previously [42]. FGF21 ERR response element mut-Luc (^{-1032bp}CAAGGTGCTT^{-1022bp} to ^{-1032bp}CAAAATGCTT^{-1022bp}) and FGF21 ERRE mut-Luc were generated using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). ERRα, ERRβ, and ERRγ constructs were described previously [43]. All plasmids used were confirmed by complete sequence analysis.

Recombinant adenoviruses

Ad-GFP, Ad-FLAG-ERRγ, Ad-US, and Ad-shERRγ were described previously [14]. All viruses were purified via CsCl₂.

Cell culture and transient transfection assays

HepG2 (human hepatoma cells) and 293T (human embryonic kidney cells) cells were obtained as described previously [44]. AML12 cells (mouse immortalized hepatocytes) were cultured in DMEM/F-12 medium (Gibco-BRL, Grand Island, NY, USA) supplemented with insulin-transferrin-selenium (Gibco-BRL), dexamethasone (40 ng/ml; Sigma, St. Louis, MO, USA), and antibiotics in a humidified atmosphere containing 5% CO₂ at 37°C. Transient transfections were conducted using LipofectAmine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The cells were treated with 10 μM GSK5182 unless noted otherwise. After 48 h of transfection, the cells were harvested, and luciferase activity was measured and normalized to β-galactosidase activity.

Culture of primary hepatocytes

Mouse primary hepatocytes were isolated from C57BL/6J or CB1^{-/-} mice (male, 20–22 g) by collagenase perfusion [45]. Rat primary hepatocytes were prepared from Male 8-week-old Sprague-Dawley rats (Damul Science, Daejeon, Korea) by a collagenase perfusion method, as described previously [46]. After being allowed to adhere for 12 h, cells were infected with the indicated adenoviruses for overexpression or knockdown. Hepatocytes were treated with 10 μM ACEA and 10 μM GSK5182.

RNA isolation and analysis

Total RNA was isolated using TRIzol reagent (Invitrogen), in accordance with the manufacturer's instructions, and real time quantitative PCR (qPCR) analysis was conducted using the following primers: ERRγ (mouse/human), 5'-AAGATCGACACATTGATTCCAGC-3' (Forward) and 5'-CATGGTTGAACTGAATTCCCAC-3' (Reverse); FGF21 (mouse), 5'-CTGCTG GGGGTCTACCAAG-3' (Forward) and 5'-CTGCGCCTACCACTGTTCC-3' (Reverse); and FGF21 (human), 5'-GGGATGTGGAGCTGGAAGTG-3' (Forward) and 5'-TGGACCAGGA AGGACTCAC-3' (Reverse). All data were normalized to β-actin (mouse/human) expression, which was determined using 5'-TCTGGCACCACACCTTCTAC-3' (Forward) and 5'-TCGTA GATGGGCACAGTGTGG-3' (Reverse) primers.

Western blot analysis

Mouse liver tissue or cultured cells were lysed with RIPA buffer and subjected to immunoblot analysis as described previously [47]. The membranes were probed with anti-ERRγ (Cell Signaling Technology, Danvers, MA, USA; diluted 1:1000), anti-β-actin (AbFrontier, Seoul, Korea; diluted 1:5000) [24] and anti-FGF21 (Abcam, Cambridge, UK; diluted 1:1000) [48] antibodies.

Measurement of FGF21 levels

Total FGF21 was extracted from cell culture medium or mouse serum. FGF21 was analyzed with a Quantikine FGF21 ELISA kit (R&D Systems, Minneapolis, MN, USA).

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY, USA). Immunoprecipitation was performed using an anti-ERR γ antibody or IgG (as a negative control). After recovery of DNA, qPCR was performed using primers encompassing the *FGF21* promoter region. The primers used for PCR were as follows: -1.95 kb/-1.75 kb, 5'-TGGGTTCTCTGACTTGCACG-3' (Forward) and 5'-CTACTCCCAGAGCATCTAGC-3' (Reverse); and -0.95 kb/-0.75 kb, 5'-ACTCCTCTTACACACTGCTG-3' (Forward) and 5'-TGGGATCTAGCTCTTGGGTC-3' (Reverse).

Statistical analyses

The results are presented as mean \pm SD. Statistical differences in one factor between two groups were determined using the unpaired Student's t test. Multiple-group comparisons were made using ANOVA. All analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA).

Results

The hepatic *FGF21* mRNA level is increased by ACEA, a CB1 receptor agonist

Previous reports demonstrated that activation of the CB1 receptor induces *CREBH* and *ERR γ* gene expression and *CREBH* regulates *FGF21* promoter activity [23, 24, 39]. Therefore, we hypothesized that *ERR γ* may regulate CB1 receptor-mediated *FGF21* gene expression. We used ACEA, a CB1 receptor selective agonist, for CB1 receptor activation [21]. ACEA treatment led to significant increases in both *ERR γ* and *FGF21* mRNA levels within 1 h and reached a maximum level at 6 h in HepG2 cells and at 3 h in rat primary hepatocytes and AML12 cells (Fig 1A–1C). In mouse primary hepatocytes, ACEA also significantly increased *ERR γ* and *FGF21* mRNA levels. However, ACEA had no effect on *ERR γ* and *FGF21* mRNA levels in CB1^{−/−} mouse primary hepatocytes (Fig 1D). Together with this direct effect of ACEA on hepatocytes, we measured hepatic *ERR γ* and *FGF21* mRNA levels in ACEA-injected C57BL/6J mice. ACEA significantly increased *ERR γ* and *FGF21* mRNA levels in mouse liver in a time-dependent manner (Fig 1E). These results demonstrate that ACEA increases *ERR γ* and *FGF21* mRNA levels.

Hepatic FGF21 is induced by ACEA

To determine whether the increases in *ERR γ* and *FGF21* mRNA levels are correlated with protein levels, western blot analysis was performed to measure FGF21 protein after ACEA treatment in HepG2 and AML12 cells. ACEA significantly increased FGF21 protein levels (Fig 2A and 2B). ACEA also induced significant increases in the FGF21 protein level in mouse liver (Fig 2C). We also examined ACEA-induced FGF21 secretion levels in AML12 cells, rat primary hepatocytes, and serum of ACEA-injected mice. ACEA treatment led to significant increases in FGF21 secretion (Fig 2D–2F). These results indicate that the CB1 receptor-specific agonist ACEA increases the protein level and secretion of FGF21.

Overexpression of ERR γ increases hepatic *FGF21* expression

Our previous study demonstrated that ethanol or ACEA induces *ERR γ* gene expression through CB1 receptor signaling in the liver [24]. In this study, we found that ACEA-mediated *ERR γ* and *FGF21* expression (Figs 1 and 2). Therefore, we examined whether ERR γ regulates *FGF21* expression by overexpression of ERR γ using Ad-ERR γ in HepG2 cells, AML12 cells, and mouse primary hepatocytes. Overexpression of ERR γ markedly increased *FGF21* mRNA levels in HepG2 cells, AML12 cells, and mouse primary hepatocytes (Fig 3A–3C). Ad-ERR γ -infected mouse liver also showed significant increases in hepatic *FGF21* mRNA (Fig 3D). To verify the effect of ERR γ on *FGF21* secretion, we measured *FGF21* levels in both AML12 cell

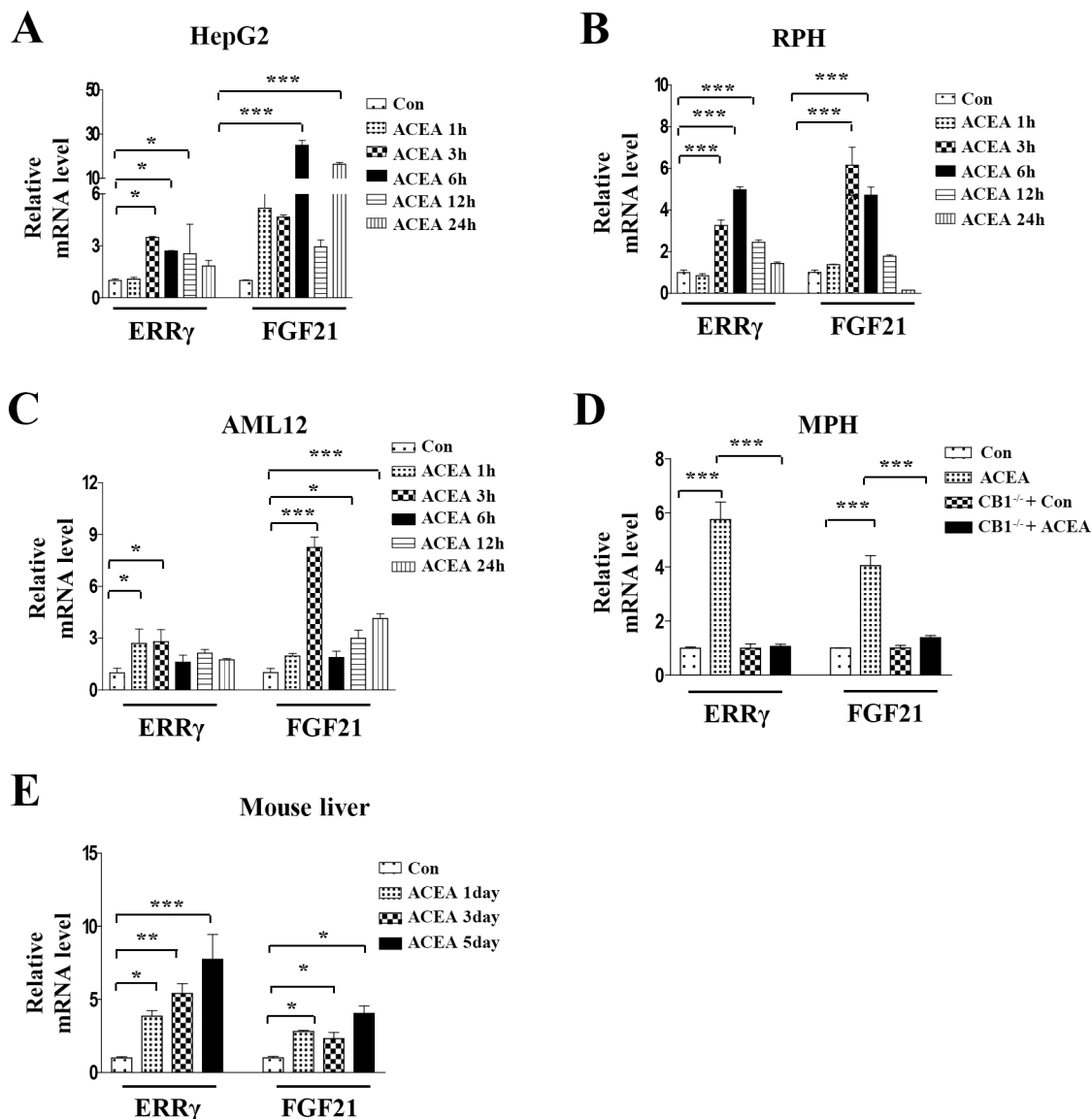


Fig 1. ACEA induces *FGF21* gene expression. (A–C) HepG2 cells, rat primary hepatocytes (RPH), and AML12 cells were treated with ACEA (10 μ M) for the indicated time periods. (D) Wild-type or CB1^{-/-} mouse primary hepatocytes (MPH) were treated with ACEA (10 μ M) for 3 h. (E) Mice were treated with ACEA (10 mg/kg) for the indicated number of days. Livers were harvested for mRNA analysis. (A–E) *FGF21* and *ERR γ* mRNA levels were measured by quantitative qPCR analysis and normalized to *actin* mRNA levels. All data are the means \pm standard errors of at least three independent experiments. * p < 0.05; ** p < 0.01; *** p < 0.001 by one-way ANOVA.

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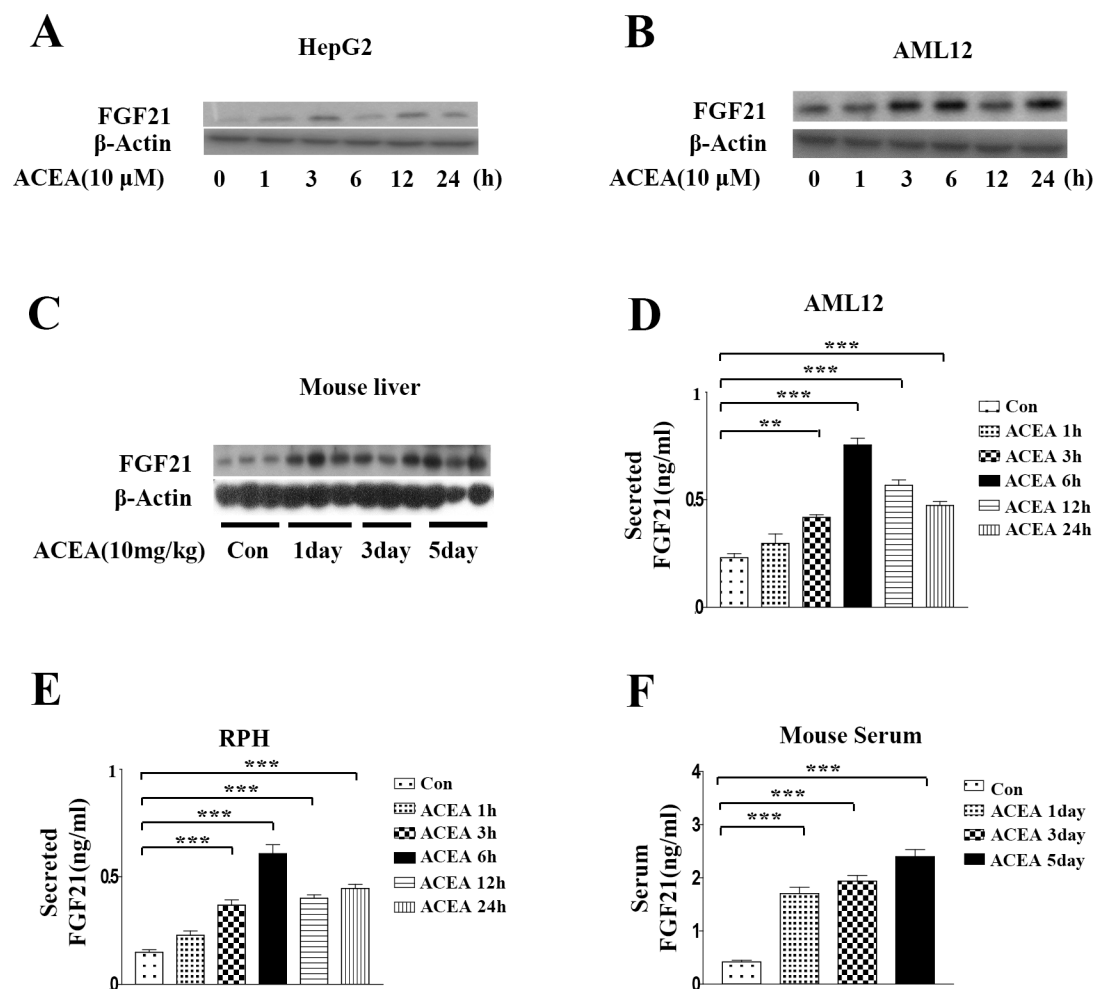


Fig 2. ACEA increases FGF21 protein levels. (A–C) Whole cell lysates of ACEA-treated HepG2 cells and AML12 cells and livers of ACEA-treated intact mice were harvested for western blot analysis. (D–E) AML12 cells and rat primary hepatocytes were treated with ACEA (10 μM) for the indicated time periods. Culture media were collected for FGF21 secretion analysis. (F) Mice were treated with ACEA (10 mg/kg) for the indicated number of days. Serum was obtained for FGF21 secretion analysis. All data are the means ± standard errors of at least three independent experiments. ** $p < 0.01$; *** $p < 0.001$ by one-way ANOVA.

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culture medium and mouse serum after ERR γ overexpression. Ad-ERR γ increased FGF21 levels in the culture medium and mouse serum (Fig 3E and 3F). These results suggest that ERR γ is a regulator of *FGF21* expression and secretion.

Knockdown of ERR γ decreases ACEA-mediated FGF21 expression

To evaluate the direct effect of ERR γ on hepatic *FGF21* gene expression through the CB1 receptor, we examined ACEA-induced *FGF21* expression after knockdown of ERR γ using an adenovirus harboring ERR γ -targeting shRNA (Ad-shERR γ). The ACEA-induced *FGF21* mRNA level was dramatically reduced by knockdown of ERR γ in HepG2 cells, AML12 cells, and mouse primary hepatocytes (Fig 4A–4C). Similar to the results in hepatocytes, ACEA-induced *FGF21* expression was significantly reduced in Ad-shERR γ -infected mouse liver (Fig 4D). Furthermore, ACEA-induced FGF21 secretion in culture media of AML12 cells was significantly decreased by shERR γ . Consistent with this finding, the ACEA-induced FGF21 level in mouse

serum was also significantly reduced by knockdown of ERR γ (Fig 4E and 4F). These results demonstrate that ERR γ is a key regulator of ACEA-mediated *FGF21* gene expression and secretion.

ERR γ activates the *FGF21* gene promoter activity

To explain the role of ERR γ in ACEA-mediated induction of *FGF21* gene expression, we investigated whether ERR γ directly induces *FGF21* gene transcription. First, we examined ACEA-

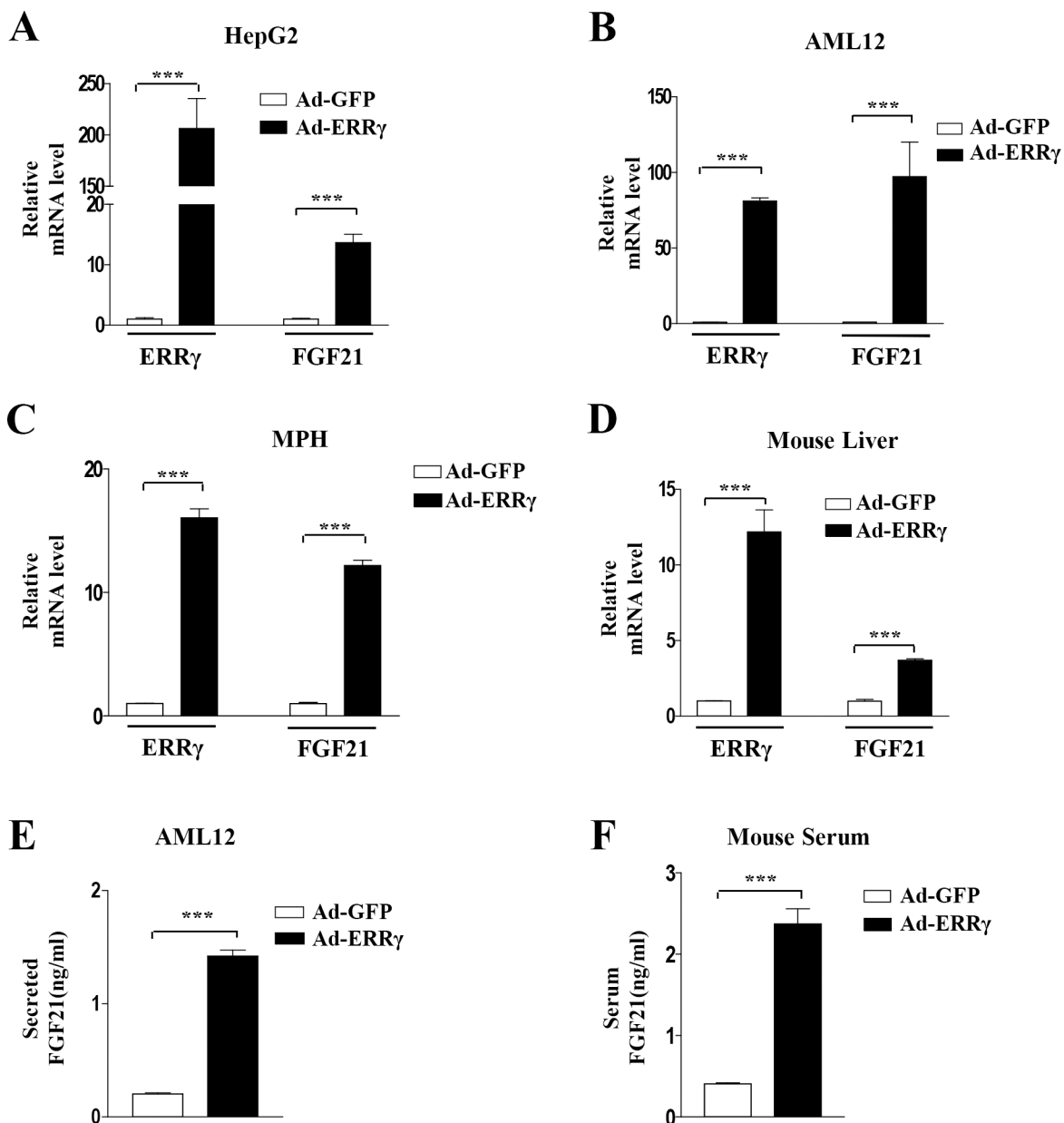


Fig 3. ERR γ overexpression induces *FGF21* gene expression. (A–C) HepG2 cells, AML12 cells, and mouse primary hepatocytes (MPH) were infected with Ad-GFP and Ad-ERR γ . (D) Ad-GFP or Ad-ERR γ was injected into male C57BL/6J mice via the tail vein. Mice were sacrificed at 5 days after injection. (A–D) *FGF21* and *ERR γ* mRNA levels were measured by quantitative qPCR analysis and normalized to *actin* mRNA levels. (E) Culture media of adenovirus-infected AML12 cells was obtained for FGF21 secretion analysis. (F) Ad-GFP or Ad-ERR γ was injected via the tail vein into male C57BL/6J mice. Serum from these mice was analyzed for FGF21 secretion. All data are the means \pm standard errors of at least three independent experiments. *** p < 0.001 by Student's t-test.

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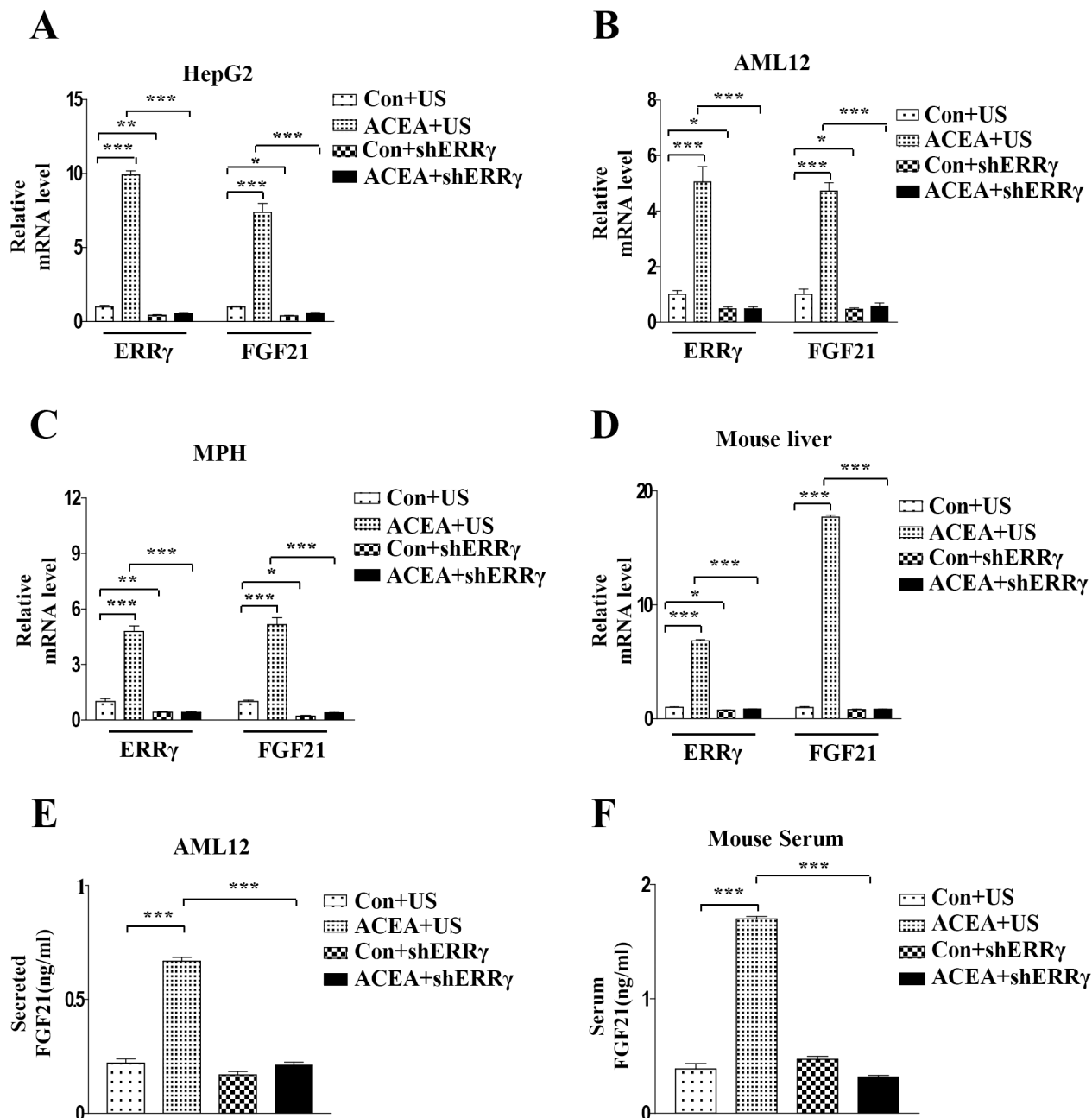


Fig 4. Knockdown of ERR γ decreases ACEA-mediated induction of *FGF21* gene expression. (A–C) HepG2 and AML12 cells were infected with Ad-US or Ad-shERR γ for 36 h and then treated with ACEA (10 μ M) for 3 h. (D and F) Ad-US and Ad-shERR γ were injected into male C57BL/6J mice via the tail vein ($n = 3–4$ per group). Four days after these injections, mice were treated for 3 days with ACEA (10 mg/kg). Serum was collected for FGF21 secretion analysis. (A–D) *FGF21* and *ERR γ* mRNA levels were measured by quantitative qPCR analysis and normalized to *actin* mRNA levels. (E) AML12 cells were infected with Ad-US or Ad-shERR γ for 36 h and then treated with ACEA (10 μ M) for 3 h. Culture media was collected for FGF21 secretion analysis. All data are the means \pm standard errors of at least three independent experiments. * $p < 0.05$; *** $p < 0.001$ by one-way ANOVA.

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induced *FGF21* promoter activity by knockdown of ERR γ using Ad-shERR γ . ACEA-induced *FGF21* promoter activity was reduced by knockdown of ERR γ in AML12 cells (Fig 5A). Next, a reporter assay with co-transfection revealed that ERR γ specifically augmented the mouse *FGF21* promoter activity, whereas ERR α and ERR β had no effect (Fig 5B). On the other hand,

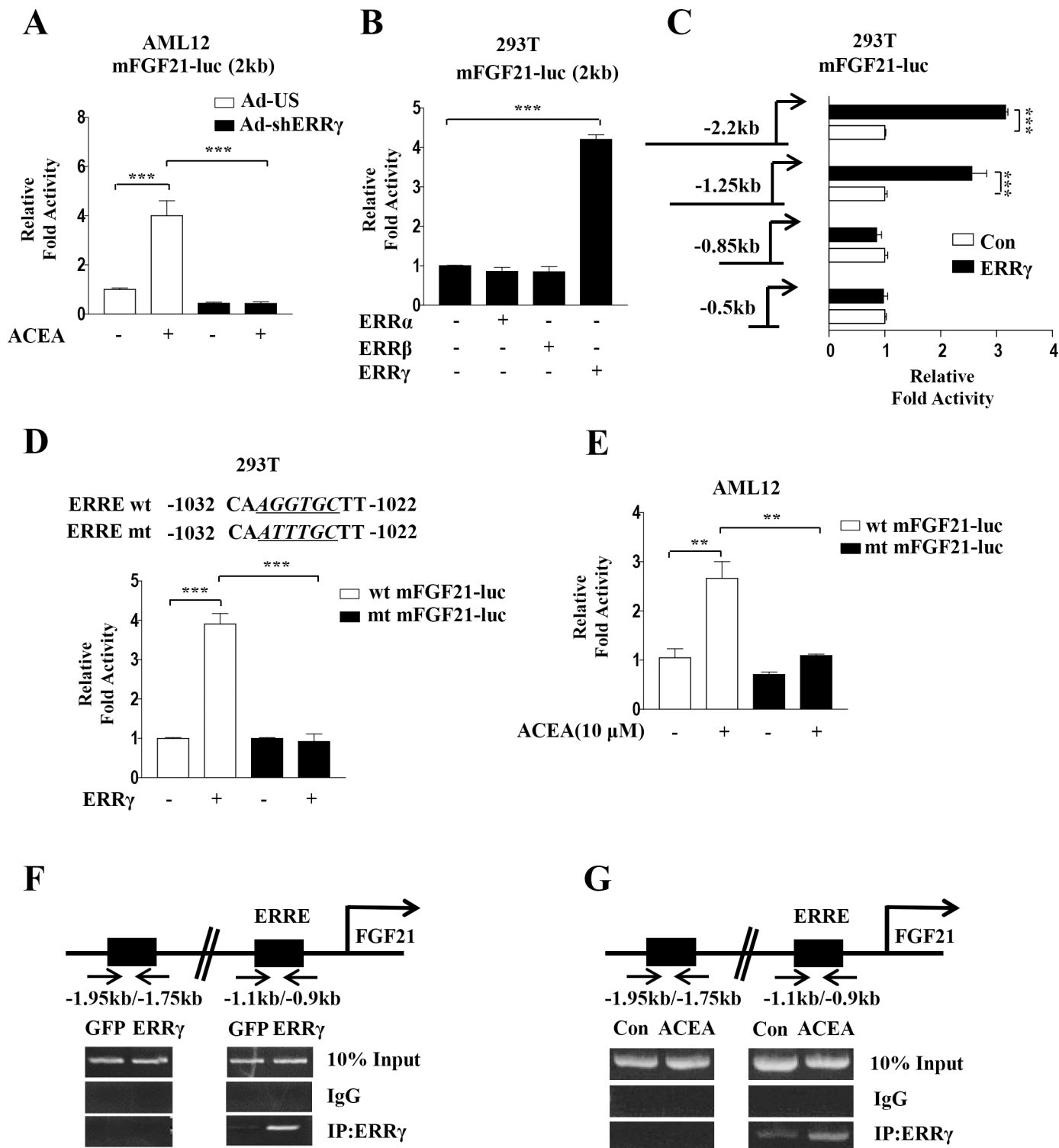


Fig 5. ERRγ activates mouse *FGF21* gene promoter activity. (A) AML12 cells were transfected with mFGF21-Luc and then treated with ACEA for 3 h. (B) 293T cells were transfected with mFGF21-Luc along with expression vectors for ERRα, ERRβ, and ERRγ. (C) 293T cells were co-transfected with deletion mutants of mFGF21-Luc and ERRγ. (D) 293T cells were co-transfected with mFGF21-Luc, mFGF21 ERRE mut-Luc, and ERRγ. The alignment of potential ERRE sequences in the human and mouse *FGF21* promoters is shown (top). (E) AML12 cells were transfected with mFGF21-Luc or mFGF21 ERRE mut-Luc and treated with ACEA for 3 h. (F-G) ChIP assay analysis was performed with Ad-GFP- or Ad-ERRγ-infected AML12 cells and ACEA treated AML12 cells. Cell extracts were immunoprecipitated with an anti-ERRγ antibody. Purified DNA samples were employed for PCR with primers that bind to the ERRE (-1.1 kb to -0.9 kb) and a distal site (-1.95 kb to -1.75 kb) in the *FGF21* gene promoter. All data are the means ± standard errors of at least three independent experiments. **p < 0.01; ***p < 0.001 by one-way ANOVA.

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serial deletion of the *FGF21* promoter showed that the region harboring -1.25 kb to -0.85 kb of the *FGF21* promoter was activated by ERR γ , and a putative ERR γ -binding motif (AGGTGC, a near-consensus response element) was identified in the *FGF21* promoter (Fig 5C). To further confirm the exact ERR γ -binding site in the *FGF21* promoter, a reporter assay was performed using wild-type and point-mutated reporter constructs of the *FGF21* promoter. ERR γ -dependent *FGF21* promoter activity was significantly decreased in the ERRE-mutated reporter construct (Fig 5D).

Next, we examined the effect of ACEA on the ERRE in the mouse *FGF21* promoter. *FGF21* promoter activity was significantly increased by ACEA. However, this ACEA-mediated *FGF21* promoter activity was decreased in ERRE-mutated reporter constructs (Fig 5E), suggesting that ACEA-dependent *FGF21* promoter activation is mediated by ERR γ . To confirm the direct binding of ERR γ to the endogenous *FGF21* promoter, ChIP assays were performed in AML12 cells. Overexpressed ERR γ and ACEA-induced endogenous ERR γ were recruited to the ERR γ consensus binding site in the *FGF21* promoter, but not to the upstream region lacking an ERRE (Fig 5F and 5G). Overall, these data suggest that activation of the hepatic CB1 receptor-induced ERR γ occupancy of the *FGF21* promoter.

GSK5182 inhibits CB1 receptor-induced *FGF21* expression and *FGF21* secretion

As an ERR γ inverse agonist, GSK5182 is used to inhibit ERR γ transactivation and its target gene expression [12, 13]. First, we examined the effect of GSK5182 on ACEA-mediated *FGF21* promoter activation. Increased *FGF21* promoter activity by ACEA was significantly decreased by GSK5182 (Fig 6A). To further clarify the direct effect of ERR γ on CB1 receptor-mediated *FGF21* expression, AML12 cells were treated with ACEA in the presence or absence of GSK5182. GSK5182 dramatically decreased *FGF21* gene expression in ACEA-treated HepG2 cells, AML12 cells, and mouse primary hepatocytes (Fig 6B–6D). In mice, the ACEA-treated group showed significant increases in ERR γ and *FGF21* gene expression, while GSK5182 significantly suppressed the ACEA-mediated *FGF21* mRNA level (Fig 6E). We also examined *FGF21* secretion by ACEA in the presence or absence of GSK5182. GSK5182 significantly decreased ACEA-mediated *FGF21* secretion in the cell culture medium (Fig 6F). In addition, the increased serum level of *FGF21* induced by ACEA was also significantly reduced by GSK5182 treatment in mice (Fig 6G). Finally, we examined the effect of GSK5182 on *FGF21* gene expression in a chronic alcohol-exposed C57BL6 mice model. Chronic alcohol exposure significantly increased hepatic ERR γ and *FGF21* gene expression. The increase in *FGF21* gene expression was decreased by GSK5182 treatment (Fig 6G). Taken together, these results indicate that the ERR γ inverse agonist GSK5182 inhibits CB1R-mediated *FGF21* expression and secretion.

Discussion

FGF21 and ERR γ play important roles in hepatic metabolism. In this study, we revealed that ERR γ contributes to CB1 receptor-induced *FGF21* gene expression. Activation of the hepatic CB1 receptor induced ERR γ and *FGF21* gene expression, and knockdown of ERR γ gene expression prevented CB1 receptor-induced *FGF21* gene expression. In addition, ERR γ induced *FGF21* gene promoter activity by directly binding to the *FGF21* promoter. Moreover, CB1 receptor-induced *FGF21* gene expression was inhibited by the ERR γ inverse agonist GSK5182.

FGF21 is regulated by the membrane receptor GHR via the JAK2-STAT5 pathway [38]. Until now, the other membrane receptor signaling pathways regulating *FGF21* expression were largely unknown. In the present study, we demonstrated that activation of the hepatic CB1

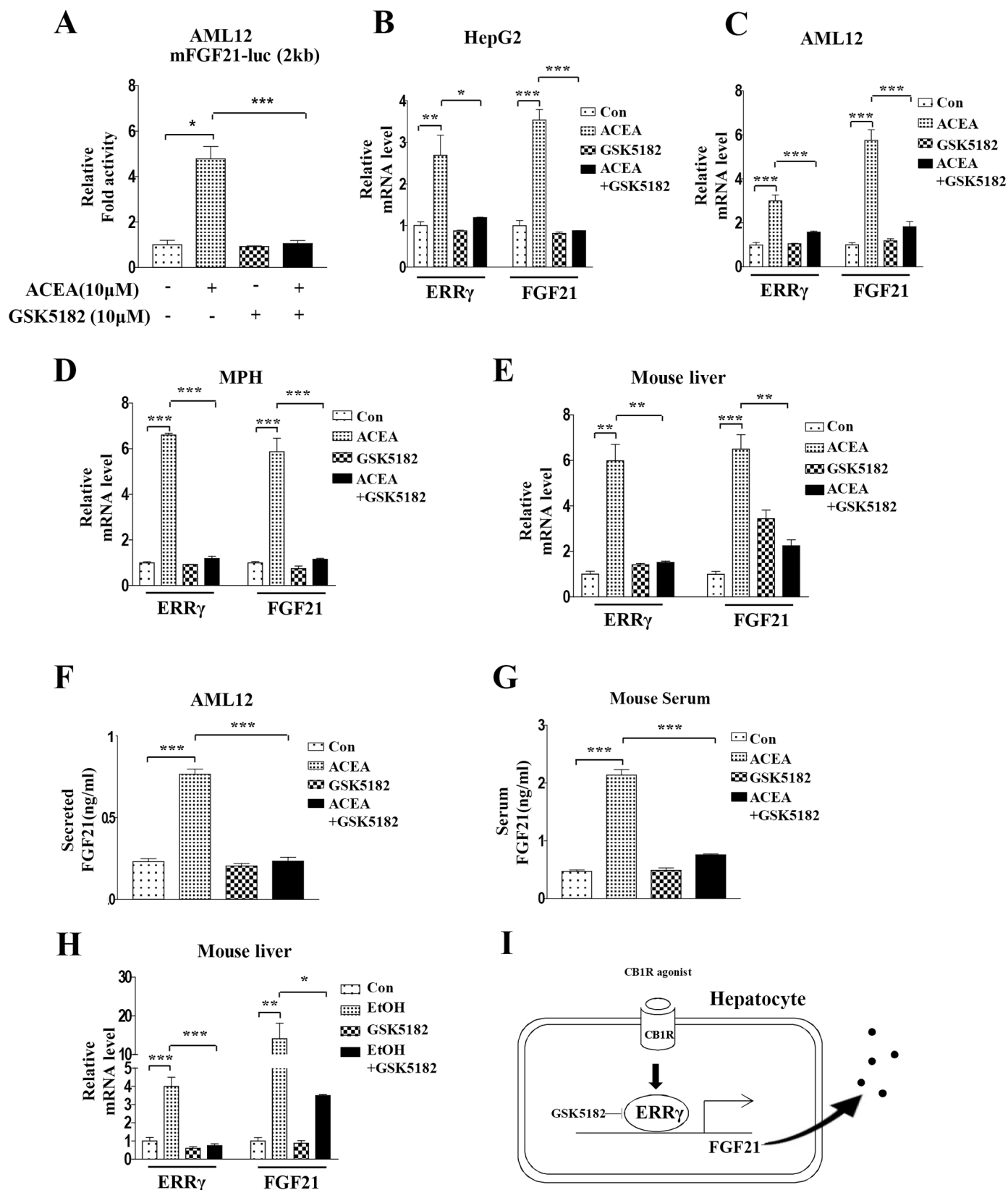


Fig 6. GSK5182 inhibits ACEA-mediated induction of *FGF21* gene expression. (A) AML12 cells were transfected with mFGF21-Luc and treated with ACEA (10 μ M) for 3 h with or without GSK5182 (10 μ M). (B–D) HepG2 cells, AML12 cells, and mouse primary hepatocytes (MPH) were treated with ACEA (10 μ M) for 3 h with or without GSK5182 (10 μ M). (E and G) GSK5182 (40 mg/kg) was administered to male C57BL/6J mice ($n = 3$ –4 per group) daily by intraperitoneal injection for 4 days. ACEA (10 mg/kg) was also given by intraperitoneal injection daily during the final 3 days. (A–D) *FGF21* and *ERR γ* mRNA levels were measured by qPCR analysis and normalized to *actin* mRNA levels. (F) AML12 cells were treated with ACEA (10 μ M) for 3 h with or without GSK5182 (10 μ M). Culture media was recovered for FGF21 secretion analysis. (G) Male C57BL/6J mice ($n = 3$) were treated with ACEA (10 mg/kg) with and without GSK5182 (40 mg/kg) daily for 3 days. Serum was analyzed for FGF21 secretion. (H) Male C57BL/6J mice ($n = 5$ per group) were fed an alcohol-containing diet for 4 weeks and GSK5182 (40mg/kg once daily) was given by oral gavage for the final 2 weeks of alcohol feeding. (I) Schematic diagram of ERR γ -mediated *FGF21* gene expression. GSK5182 inhibits activation of *FGF21* gene expression and FGF21 secretion mediated by increased ERR γ caused by activation of the hepatic CB1 receptor. All data are the means \pm standard errors for at least three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by one-way ANOVA.

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receptor induced *FGF21* gene expression via the induction of ERR γ gene expression. ACEA was used to activate the CB1 receptor because ACEA is the most selective synthetic agonist of the CB1 receptor. Activation of the hepatic CB1 receptor induced FGF21 expression and secretion (Figs 1 and 2). Several nuclear receptors are involved in regulation of *FGF21* gene expression. To date, most studies have focused on transcription factors that control transcription in the -1200bp and -1000bp region of the *FGF21* gene. FGF21 is induced by PPAR α in the liver during fasting by direct binding to the PPAR response element (-1093bp and -1057bp) in the *FGF21* promoter [31, 49]. *FGF21* gene expression is induced by Nur77 during fasting via direct binding of Nur77 to the NGFI-B response element (-1282bp and -1248bp) in the promoter [50]. In addition, FXR, ROR α , and LXR regulate *FGF21* gene expression through direct binding to the *FGF21* promoter [35, 37, 51]. Here, we found that overexpressed ERR γ induced *FGF21* gene expression (Fig 3), and knockdown of ERR γ reduced CB1 receptor-induced FGF21 levels (Fig 4). Moreover, the present study provides an alternative mechanism by which *FGF21* promoter activity is mediated by hepatic ERR γ via the putative ERRE sequence (-1032bp and -1022bp) (Fig 5). Previously, it was reported that rosiglitazone, a PPAR γ agonist, induces FGF21 in white adipose tissue, but not in the liver. All trans-retinoic acid, an endogenous ligand of RAR β , induces *FGF21* gene expression in liver tissue [36].

In this study, we demonstrated that GSK5182 inhibited hepatic CB1 receptor-induced *FGF21* gene expression via specific inhibition of the transcriptional activity of ERR γ . We also revealed that the hepatic CB1 receptor is a new membrane receptor regulating FGF21 gene expression, and that CB1 receptor-induced ERR γ is a new upstream regulator of *FGF21* gene expression. Finally, ERR γ inverse agonist GSK5182 inhibited *FGF21* gene expression in response to CB1 receptor activation. Previously, we reported that ERR γ regulates gluconeogenesis in the liver [11, 32]. ERR γ directly binds to the phosphoenolpyruvate carboxykinase (PEPCK) promoter and induces *PEPCK* gene expression. FGF21 increases hepatic gluconeogenesis through PGC-1 α -mediated gluconeogenic gene expression [32]. Despite direct regulation of hepatic gluconeogenesis by ERR γ , ERR γ -induced FGF21 could be another pathway that regulates hepatic gluconeogenesis. In addition, activated CB1 receptor-induced ERR γ mediates gene expression of CYP2E1, which is a key enzyme generating alcohol-induced reactive oxygen species (ROS) in the liver [24]. In this study, hepatic ERR γ and *FGF21* mRNA levels were significantly increased in the chronic alcohol-exposed mice liver (Fig 6H). Other studies suggest that APAP induces hepatic ROS generation and FGF21 production. APAP-induced FGF21 protects against hepatotoxicity via inducing antioxidant gene expression [33]. Furthermore, previous study suggests that FGF21 markedly reduces alcohol preference in mice [52]. This effect requires the FGF21 co-receptor beta-klotho in the central nervous system. Previous studies suggest that the hepatic CB1 receptor contributes to obesity in a diet induced obesity (DIO) mouse model by increasing free fatty acid synthesis [18]. Paradoxically, recombinant murine FGF21 treatment reverses hepatic steatosis by increasing energy expenditure in DIO mice [53].

Our previous study revealed that ERR γ gene expression is also higher in DIO mice [54]. Therefore, we speculate that intrinsic FGF21 might be involved in CB1 receptor signaling-mediated free fatty acid synthesis. However, excessive increases in FGF21 induced by ERR γ may have a beneficial effect on hepatic steatosis. The FGF21 level is higher under stressful conditions, including starvation and high fat diet, and FGF21 improves metabolic disorder under these conditions. Therefore, our study suggests that CB1 receptor-induced ERR γ contributes to the FGF21-mediated compensatory mechanism to oppose CB1 receptor-mediated diet-induced obesity.

Conclusions

Overall, our results reveal that ERR γ , induced via activation of the hepatic CB1 receptor, is a regulator of hepatic *FGF21* gene expression and secretion. GSK5182 inhibited CB1 receptor-mediated *FGF21* expression and secretion (Fig 6I), confirming that FGF21 is a direct target of ERR γ . In addition, chronic alcohol exposed mice showed increased FGF21 gene expression as a result of the induction of ERR γ gene expression. The identification of ERR γ as a mediator of FGF21 expression will increase current understanding of the mechanisms involved in controlling hepatic metabolism by FGF21.

Supporting Information

S1 Fig. Western blot (uncropped) for Fig 2A.

(DOCX)

S2 Fig. Western blot (uncropped) for Fig 2B.

(DOCX)

S3 Fig. Western blot (uncropped) for Fig 2C.

(DOCX)

S4 Fig. Electrophoresis gel of the ChIP assay (uncropped) for Fig 5F.

(DOCX)

S5 Fig. Electrophoresis gel of the ChIP assay (uncropped) for Fig 5G.

(DOCX)

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Author Contributions

Conceived and designed the experiments: YSJ HSC. Performed the experiments: YSJ YSL KSK. Analyzed the data: YSJ. Contributed reagents/materials/analysis tools: RAH MSL IKL WIJ CHL JML DKK YHK SHK SJC JK. Wrote the paper: YSJ HSC.

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